Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2022; 11(4): 283-294
Received: 23-05-2022
Accepted: 29-06-2022

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HPTLC fingerprinting reveals leaf and roots phytochemical variability in developmental stages of *Withania somnifera*

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DOI: https://doi.org/10.22271/phyto.2022.v11.i4d.14473

Abstract
Background: *Withania somnifera* has been the foundation of numerous contemporary medicines today for treatment of various diseases. However, phytochemical variability profile is lacking in the root and leaf organs.
Aim: This study was aimed to elucidate the phytochemical profile in seedling, vegetative and reproductive stages of leaf and roots in *W. somnifera* using High performance thin layer chromatography (HPTLC) fingerprinting.
Methods: The HPTLC with dual wavelength UV (254/366 nm) was used for the analysis of phytochemical variation in leaf and root methanolic extracts of *W. somnifera*.
Results: HPTLC chromatograms in 366 nm, 254 nm, and normal day light, seedling, vegetative and reproductive stages leaf and root exhibited bands variability. At 450 nm leaf HPTLC fingerprints in seedling stage showed five peaks with Rf values in the range of 0.42 to 0.90, vegetative stage with nine peaks of Rf values in range of 0.10 to 0.89, and reproductive stage with six peaks of Rf values in range of 0.30 to 0.92. In root showed six peaks with Rf values in range of 0.00 to 0.82 in seedling stage, ten peaks with Rf values in range of 0.00 to 0.96 in vegetative stage, seven peaks with Rf values in range of 0.01 to 0.91 in reproductive stage. In roots, higher peak areas were found at the reproductive stage (3.33%) compared to vegetative stage (1.65%).
Conclusion: The comparative HPTLC fingerprints of leaves and roots in *W. somnifera* presented phytochemical variability in leaf and root that varied with developmental stages.

Keywords: HPTLC fingerprinting, *Withania somnifera*, leaf, root, phytochemicals, herbal medicine

1. Introduction
Herbal medicines have been actively utilized in the health care system of Indian history since immemorial time. But its growth as an industry is still not explored to its full potential (Ekor, 2014; Petrovska, 2012; Pandey *et al*., 2013) [1, 2, 3]. However, rates of its production have been increased noticeably in the past few years (Jang *et al*., 2017) [4]. Hence, there is a need to transmute the herbal medicine into a vibrant scientifically authenticated and evidence based industrial products, based on deep understanding of traditional medicine and available research (Mohiuddin, 2019) [5]. In fact, it is important to use methods for rapid and precise identification and estimation of specific stage of medicinal plants and the essential phytoconstituents, as the qualitative and quantitative objectives to assess the genuineness and crucial quality (Balekundri and Mannur, 2020; Kira nmai, 2016) [6, 7]. The estimation and evaluation of herbal medicinal plants, its phytochemicals through an accurate method would offer to find the true essential phytochemicals in herbs used for therapeutic purposes and provide a precise foundation of herbal medicine effectiveness (Pan *et al*., 2013; Jasemi *et al*., 2020) [8, 9].
The identification and estimation can be achieved through various analytical techniques including HPTLC (Attimarad *et al*., 2011; Patel *et al*., 2011) [10, 11]. Such analytical techniques will ascertain the presence of essential phytochemical constituents and quantify their concentrations (Sonia *et al*., 2017) [12]. HPTLC offers many advantages over other various analytical methods of chromatographic techniques. It can present the results in an image, autonomous, reliable, easier to handle, many samples for parallel analysis, low in experimental expenses, rapid presentation of the results, and detection sensitivity of phytoconstituents in picogram (pg) and nanogram (ng) concentrations (Kharat *et al*., 2017; Nikolin *et al*., 2004; Bairy, 2015) [13, 14, 15].

In the herbal medicinal research, HPTLC is widely used to analyse the presence of phytochemicals (Shinde *et al*., 2011) [16]. Moreover, HPTLC analysis integrated with digital scanning and profiling offers precise measurable assessment of Rf values of herbal samples into chromatogram peaks with defined parameters as scanning densitometry documentation, counting observance of intensity with corresponding Rf values (Coran and Mulas, 2012; Gallo *et al*., 2008) [17, 18].

Fingerprinting with HPTLC is a reasonable choice for effective quality assessment of Indian Traditional Medicine (ITM) (Senguttuvan and Subramaniam, 2019) [19]. The improved HPLC fingerprinting is not only a substitute analytical method for validations but an approach to present and identify the differences in patterns of types of phytochemicals distributed in the herbal medicinal plants (Ram *et al*., 2011) [20].

Ashwagandha (winter cherry), is an important medicinal herb in Indian Ayurveda, for more than 3000 years used as indigenous medicinal herb (Murthy *et al*., 2010) [21] and cultivated (Kotteswari *et al*., 2018) [22]. Indian ginseng is the other name given based on its reported properties like, sedative, tonic, adaptogens, hypnotic, analgesic, diuretic, antipyretic, abortifacient, and anti-inflammatory (Murthy *et al*., 2010) [23]. Numerous products of Ashwagandha plays prominent roles in the Indian market with its products containing diverse therapeutic benefits (Bharti *et al*., 2016) [24]. Both traditional and modern formulations products of Ashwagandha are available in various forms in the market (Gill *et al*., 2019) [25].

Considering the demand for herbal medicinal products, there is a need for identifying the plant and its parts with respect to its developmental stage with sophisticated and swift analytical techniques to identify differences in the phytochemicals for the medicinal producers (Mukherjee *et al*., 2021; Fitzgerald *et al*., 2020; Abubakar and Haque, 2020) [26, 27, 28]. Hence, in this study, HPTLC fingerprinting profile was carried to identify and estimate the phytochemical variability in the leaf and root organs of seedling, vegetative and reproductive stages in *W. somnifera*.

2. Materials and Methods

2.1. Collection of the Plant Material

The seeds of *W. somnifera* were procured from Zooqa Herbs Chennai, Tamil Nadu. The seeds were sowed and grown in the natural soil condition at the Department of Genetics, Osmania University, Hyderabad, Telangana. The identification and authentication of plants were done in the Department of Botany, Osmania University, Hyderabad, Telangana. Further, the seeds were sown, and plants were grown in natural soil for further experimental use.

2.2. Methanol extraction

The leaves and roots were collected during seedling, vegetative and reproductive stages for the methanolic extraction of phytochemicals. The collected plant samples were washed thoroughly with the tap water and then once with sterile distilled water. Later, the plant samples were dried under shade in a dry space. It was then grinded to powder in a sterilized clean mortar and pestle. This powder was then used for extraction of phytochemicals and the left-over powder was stored in an airtight glass bottle for further use.

2.3. Preparation of the extracts

Twenty grams of leaf or root sample powders was used for the extraction with methanol (100 mL) using Soxhlet apparatus at hot condition. Then, using Whatman filter paper, the extracts were filtered and dehydrated. Using rotary vacuum evaporator, the extracts were dried and stored in refrigerator for further use.

2.4. HPTLC Fingerprinting Profile

The leaf and root extracts of *W. somnifera* HPTLC fingerprint profile was carried out using HPTLC system with twin trough chamber lid (10×10 cm CAMAG, Muttenz, Switzerland). The sample applicator Linomat 5 (Anchrom Enterprises (I) Pvt. Ltd, Mumbai) and the UV cabinet with dual wavelength (254/366 nm) were used for the HPTLC photo documentation (Aetron, Mumbai).

2.5. Chromatographic Conditions

Using a CAMAG Linomat 5 sample applicator (Switzerland) 250 µm thickness (E. MERCK, Darmstadt, Germany) was laid onto precoated silica gel aluminium plate 60 F_{254} (5 × 10). The samples of 10 µL (E230621 1 to 3) were dissolved in methanol and applied with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) of band with width 6 mm. Prewashing of the plate was done with methanol and treated at 110 ^{0}C for 5 minutes to activate. The saturation time of mobile phase was kept for 15 min in an optimized chamber. The run was done on the chromatogram for length of 8 cm. In a current of air, the HPTLC plate was dried with hair dryer. For the analysis, 5 × 0.45 mm slit dimensions and 20 mm/sec scanning speed were employed.

2.6. Mobile phase

The mobile phase with composition n-Hexane: Ethyl Acetate (6: 4) was used.

2.7. Calculation of Retention factor (Rf) Values

Plates were visualized in the daylight and with UV light (254/366 nm). The spots central points that appeared on chromatogram were marked with needle after each observation. The following formula for Rf was (Chatwal, 2004) used:

Rf = A/B where,

A= distance from point of application to the spot central point.

B= distance from point of application to the mobile phase front.

The scanning was done at 450 nm wavelength at which the results were documented.

3. Results

3.1. HPTLC profile of leaves

The HPTLC analysis performed on leaves at seedling, vegetative and reproductive stages is shown in Fig. 1. In the methanolic extracts of leaf the HPTLC chromatogram identified various bands under the sources of UV 366 nm, UV 254 nm, and normal day light revealing the presence of differences in the bands. HPTLC chromatograms of methanolic extracts of leaf in seedling stage exhibited two blue bands at 366 nm, two light green bands at 254 nm, one light green band and one dark green band in normal day light (Table 1). In vegetative stage, two dark blue bands at 366 nm, two light green and one dark green at 254 nm and three light green and one dark green band in normal day light. In reproductive stage two dark bands at 366 nm, one dark green, one dark blue band at 254 nm, and one dark green band in the normal day light were observed (Figure 1 and Table 1).

The data for peaks obtained by scanning the HPTLC plate at 450 nm is shown in Fig. 2. The peak variations in the densitometric HPTLC of *W. somnifera* leaf methanolic extract at seedling, vegetative and reproductive stages respectively is shown in Fig. 3, and Fig. 4. The HPTLC densitometric variations in peaks, Rf values and peak areas are depicted in Tables 2, 3 and 4 of seedling, vegetative and reproductive stages respectively. Table 2 shows the presence of various peaks in the leaf methanolic extracts of seedling stage with values of Rf 0.42, 0.51, 0.65, 0.84 and 0.90 respectively, showing presence of five peaks. Table 3 shows the presence of peaks in the leaf methanolic extracts at vegetative stage with Rf values of 0.10, 0.30, 0.50, 0.59, 0.70, 0.74, 0. 83, and 0.89 respectively, showing the presence of nine peaks. Table 4 shows the presence of peaks in the leaf methanolic extracts at reproductive stage with values of Rf 0.30, 0.40, 0.49, 0.63, 0.79, and 0.92 respectively, showing the presence of six peaks. The comparative display of the peak variation of leaf methanolic extracts from seedling, vegetative and reproductive stages in *W. sominifera*, 3D densitogram at 450 nm is shown in Figure 5.

3.2. HPTLC profile of roots

The HPTLC analysis performed on roots at seedling, vegetative and reproductive stages is shown in Fig. 6. In the methanolic extracts of roots, bands were identified under the sources of UV 366 nm, UV 254 nm, and normal day light revealing the presence of differences in the bands. The methanolic extracts of roots at seedling stage produced one fluorescent band of light red at 366 nm, and four dark blue bands at 254 nm. In vegetative stage, root extracts produced six light blue bands and two light red bands at 366 nm and five dark blue bands at 254 nm. In reproductive stage, the root extracts produced five light blue bands and one light red band at 366 nm and four dark blue band at 254 nm (Figure 6 and Table 1).

The data for peaks obtained by scanning the HPTLC plate at 450 nm is shown in Fig. 7, The peak variations in densitometric HPTLC of *W. somnifera* root methanolic extract at seedling, vegetative and reproductive stages respectively are shown in Fig. 8 and Fig. 9. The HPTLC densitometric variations in peaks, Rf values and peak areas are depicted in the Tables 5, 6 and 7 for seedling, vegetative and reproductive stages respectively. Table 5 shows the presence of various peaks in the root methanolic extracts of seedling stage with values of Rf as 0.00, 0.07, 0.39, 0.64, 0.73, and 0.82 respectively, showing the presence of six peaks. Table. 6 shows the presence of peaks in the root methanolic extracts at vegetative stage with Rf values of 0.00, 0.07, 0.32, 0.54, 0.61, 0.71, 0. 81, 0.89, and 0.96 respectively, showing the presence of ten peaks. Table. 7 shows the presence of peaks in the root methanolic extracts at reproductive stage with Rf values of 0.01, 0.13, 0.33, The comparative display of the peak variation of root methanolic extracts from seedling, vegetative and reproductive stages in *W. somnifera*, 3D densitogram at 450 nm is shown in Figure 10.

4. Discussion

Withania somnifera has been playing an important role as a part of primary medicines to treat various ailments for the large section of population in the developing countries (Dutta *et al*., 2019) [29]. Moreover, the traditional medicine use is not just restricted to developing countries, and with expanding use of herbal products in the past two decades interest of public in natural therapies has greatly increased in developed countries (Ravishankar and Shukla, 2007) [30]. The herbal medicinal products like *W. somnifera* tablets are being used as health supplements and as herbal medicine to cure some chronic health conditions (Yuan *et al*. 2016) [31]. It has proven to rejuvenate the nervous system, cure insomnia, stress, lower blood pressure and is highly effective in preventing the development of stress induced ulcers (Dongre *et al*., 2015) [32]. Moreover, the high valued roots are used to treat ailments either alone or in combination with other herbals (Bhat *et al*., 2015) [33]. Furthermore, *W. somnifera* is widely considered as organic and reliable herbal medicinal plant having less toxic than that of synthetic medicines (Umadevi, 2012) [34]. Hence, the herbal medicines based on W. *somnifera* are growing rapidly than any other alternative therapy in India (Tandon and Yadav, 2020) [35].

With the growing demands of *W. somnifera* products, the conventional ways of plant identification, phytochemical extraction, preparation of herbal products need to be replaced with more precise approaches to ensure quantity, quality, safety and consistency (Pratte *et al*., 2014) [36]. The HPTLC fingerprinting approach has been a feasible and coherent methodology for evaluating quality and herbal medicine authentication (Shaikh and Patil, 2020) [37]. Moreover, HPTLC fingerprinting peaks data can be used for quantitative and qualitative determination of detectable phytochemicals (Frommenwiler *et al*., 2019) [38]. Hence, the work was carried out to unveil the variation in phytochemical constituents and their concentrations present with respect to their developmental stages in *W. sominifera*.

The fingerprint of leaf methanolic extracts of *W. somnifera* at seedling stage exhibited two blue bands at 366 nm corresponding to the phenolic compounds caffeic, p-coumaric, two light green at 254 nm corresponding to flavonoid compound naringenin and one light green and one dark brown band at normal light indicating flavones (Stanek and Misiak, 2018) [39]. At the vegetative stage two dark blue bands at 366 nm corresponding to the phenolic compounds caffeic and p-coumaric, two light green and one dark green at 254 nm corresponding chrysin and three light green and one dark green at normal light corresponding to naringenin were exhibited (Stanek and Misiak, 2018) [39]. At the reproductive stage two dark brown bands at 366 nm, representing the phenolic compounds gallic or salicylic acids, one dark green, one dark blue band at 254 nm and one dark green band in the normal light corresponding to flavonoid compound chrysin were exhibited (Stanek and Misiak, 2018) [39].

The fingerprint of root methanolic extracts of *W. somnifera* at seedling stage exhibited phytochemical profiles with one red band at 366 nm corresponding to phenolic compound anthocyanin, four dark blue bands at 254 nm corresponding to the ferulic acid (Stanek and Misiak, 2018) [39]. At vegetative stage six light blue bands, two light red bands at 366 corresponding to steroid compounds and phenolic compound anthocyanins respectively were exhibited (Aparna and Aruna, 2014) [40] and five dark blue bands at 254 nm representing ferulic or phenolic compounds (Stanek and Misiak, 2018) [39]. At the reproductive stage five light blue bands and one light red band at 366 nm corresponding to steroid compound and phenolic anthocyanins (Aparna and Aruna, 2014) [40] and four dark blue band at 254 nm that corresponding to ferulic acids were exhibited (Stanek and Misiak, 2018) [39].

To obtain high phytochemical constituents in plant extracts, methanol is considered as an optimal solvent (Soni *et al*., 2017) [12]. In this study, variability in phytochemicals was observed at the seedling, vegetative and reproductive stages in the HPTLC profile of leaf and root methanolic extracts. The variation in colours in HPTLC chromatogram bands is attributed to variation in phytochemicals (Shetty and

Nareshchandra, 2012) [41]. In the previous HPTLC fingerprinting of methanolic extracts from available capsule made of *W. somnifera* root has confirmed the presence and absence of phytochemicals, and their concentrations (Nicoletti, 2011) [42]. However, there are no comparative HPTLC fingerprinting studies been done yet on leaf and root methanolic extracts of *W. somnifera*. Hence, this report is the first to unveil and comparatively present the variation in the phytochemical constituents of leaf and root extracts, with their developmental stages through HPTLC fingerprinting.

The number of bands in HPTLC chromatogram are comparatively more for both root and leaf methanolic extracts at vegetative stage. This might be due to biosynthesis of phytochemicals activated more during vegetative stage in the plant and may transformed to complex structures at later stages *viz*., reproductive stage (Thakur, 2020) [43]. It is observed that number of bands are more in root extracts at vegetative and reproductive stages. This may be due to transport of phytochemicals from its production sites to its storage site *viz*. root. Up to 80% photosynthetic fixed carbon in mature leaves can be exported in *W. somnifera* (Lemoine *et al*., 2013) [44]. At 450 nm the HPTLC fingerprinting profile differed in terms of peak numbers and concentrations in both the methanolic leaf and root extracts of *W. somnifera*. At seedling stage the least number of peaks were shown in HPTLC fingerprinting chromatograms analysed at 450 nm (Figures 2 and 7; Tables 2 and 5). At vegetative stage the leaf methanolic extracts differed from seedling and reproductive stages with three unique peaks having Rf 0.10, 0.70, and 0.74 in HPTLC fingerprint (Figure 3 and Table 3). It was observed that the peak at Rf value 0.30 was found to be common in the leaf methanolic extracts in both vegetative and reproductive stages with higher peak areas at the reproductive stage (3.33%) and least concentrations at the vegetative stage (1.65%) (Figures 3 and 4; Tables 3 and 4). In the root methanolic extracts the variation in the peaks is observed with higher peak areas and numbers at the vegetative stage (27.53%) and decreased concentration and lesser number of peaks at the reproductive stage (21.32%) in the HPTLC fingerprinting chromatograms analysed at 450 nm (Figures 7, 8, 9 and Tables 5, 6, 7).

The important finding in this study shows that the peak areas at respective Rf values in the methanolic extracts of root are much higher than leaf extracts in *W. somnifera* (Tables 3, 4, 6 and 7). The variation in the phytochemical constituents and their concentrations being higher in the root extract must be holding an important attribute behind the root being more health beneficial (Altemimi *et al*., 2017) [45]. Such differences and similarities in the peaks of chromatograms and concentrations may be useful to assess and correlate the medicinal properties as well. There are studies on the methanolic extracts of roots and leaf for antibacterial properties (Truong *et al*., 2019) [46]**.** The *W. somnifera* antibacterial properties are attributed to the phytochemicals combinational effect present in the methanolic extracts (Alam *et al*., 2012) [47]. Hence, unlike the previous conclusions that the particular medicinal properties are the cumulative effect of all the compounds in composite (Dharajiya *et al*., 2014) [48], The phytochemicals present or absent can primarily influence the medicinal properties at particular developmental stage of plant. Hence, the HPTLC fingerprinting analysis of leaf and root methanolic extracts of *W. somnifera* in developmental aspects can ensure a precise preconcepts for the herbal medicinal manufacturers to productively harvest the finest plants at its best developmental stage in the best seasons.

From this study it can be concluded that there is a major phytochemical variation in *W. somnifera* leaf and roots during its developmental stages *viz*. seedling, vegetative and reproductive stages. Although HPTLC is effective in comparative analysis of the qualitative evaluation of phytochemical constituents (Kamboj and Saluja, 2013) [49] the phytochemicals corresponding to its respective peaks need further identification precisely at the different retention factors of the HPTLC chromatogram. Further studies are needed to determine bioactivity lead fractionation and evaluation of peaks to reveal the nature of phytochemicals present (Palve *et al*., 2015) [50].

Table 1: HPTLC chromatogram color bands and numbers observed in normal light, 366 nm and 254 nm of leaf and root methanolic extracts in *Withania somnifera*.

Light Source	HPTLC chromatogram band color and number observed					
	Leaf			Root		
	Seedling stage	Vegetative stage	Reproductive stage	Seedling stage	Vegetative stage	Reproductive stage
Normal Light	Light green (1) Dark brown (1)	Light green (3) Dark green (1)	Dark green (1)	No bands	No bands	No bands
366 nm	Blue (2)	Dark Blue (2)	Dark brown (2)	Red (1)	Light Blue (6) Red (2)	Light blue (5) Red (1)
254 nm	Light green (2)	Light green (2) Dark green (1)	Dark green (1) Dark blue (1)	Dark blue (4)	Dark blue (5)	Dark blue (4)

Table 2: HPTLC peak parameters (Rf values, height and area) of leaf methanolic extracts from seedling stage in *W. sominifera*.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	%Area
1	0.42 Rf	6.0 AU	0.45 Rf	21.6 AU	7.80%	0.49 Rf	6.2 AU	669.4 AU	7.90%
2	0.51 Rf	11.1 AU	0.55 Rf	120.8 AU	43.55%	0.59 Rf	2.1 AU	3105.7 AU	36.66%
3	0.65 Rf	5.4 AU	0.74 Rf	57.7 AU	20.82%	0.81 Rf	0.1 AU	2882.7 AU	34.03%
4	0.84 Rf	1.9 AU	0.87 Rf	20.4 AU	7.34%	0.90 Rf	7.6 AU	503.7 AU	5.95%
5	0.90 Rf	7.7 AU	0.93 Rf	56.8 AU	20.49%	0.97 Rf	0.3 AU	1310.5 AU	15.47%

Table 3: HPTLC peak parameters (Rf values, height and area) in leaf methanolic extracts from vegetative stage in *W. sominifera.*

Peak	Start Position	Start Height	Max Position	Max height	Max %	End Position	End Height	Area	%Area
1	0.10 Rf	6.9 AU	0.13 Rf	12.7 AU	1.19%	0.15 Rf	1.7 AU	310.9 AU	0.87%
2	0.30 Rf	0.1 AU	0.35 Rf	22.8 AU	2.15%	0.37 Rf	7.7 AU	587.6 AU	1.65%
3	0.40 Rf	6.9 AU	0.47 Rf	128.4 AU	12.07%	0.50 Rf	109.5 AU	5153.0 AU	14.46%
4	0.50 Rf	110.4 AU	0.54 Rf	466.4 AU	43.85%	0.58 Rf	26.9 AU	15336.5 AU	43.04%
5	0.59 Rf	27.1 AU	0.66 Rf	189.1 AU	17.77%	0.70 Rf	50.9 AU	8136.7 AU	22.84%
6	0.70 Rf	51.3 AU	0.72 Rf	83.0 AU	7.80%	0.74 Rf	57.5 AU	2172.0 AU	6.10%
7	0.74 Rf	57.9 AU	0.76 Rf	79.0 AU	7.43%	0.81 Rf	0.3 AU	1814.9 AU	5.09%
8	0.83 Rf	0.6 AU	0.87 Rf	25.0 AU	2.35%	0.89 Rf	15.5 AU	792.9 AU	2.23%

9	0.89 Rf	16.0 AU	0.92 Rf	57.4 AU	5.39%	0.95 Rf	3.9 AU	1326.9 AU	3.72%

Table 4: HPTLC peak parameters (Rf values, height and area) of methanolic extracts from leaf of reproductive stage in *W. sominifera*.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	% Area
1	0.30 Rf	1.3 AU	0.35 Rf	11.8 AU	3.63%	0.38 Rf	1.2 AU	358.9 AU	3.33%
2	0.40 Rf	0.8 AU	0.45 Rf	17.9 AU	5.53%	0.48 Rf	6.3 AU	644.8 AU	5.99%
3	0.49 Rf	6.5 AU	0.53 Rf	14.2 AU	4.40%	0.60 Rf	0.1 AU	727.9 AU	6.76%
4	0.63 Rf	1.4 AU	0.71 Rf	94.4 AU	29.17%	0.75 Rf	16.8 AU	3496.3 AU	32.45%
5	0.79 Rf	1.5 AU	0.84 Rf	84.9 AU	26.24%	0.92 Rf	6.2 AU	3396.7 AU	31.53%
6	0.92 Rf	6.4 AU	0.95 Rf	100.4 AU	31.02%	0.98 Rf	18.0 AU	2148.8 AU	19.95%

Table 5: HPTLC peak parameters - Rf values, height and area- of methanolic extracts from roots of seedling stage in *W. sominifera*.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	%Area
1	0.00 Rf	0.6 AU	0.03 Rf	170.9 AU	53.21%	0.07 Rf	21.3 AU	2977.9 AU	41.95%
2	0.07 Rf	21.4 AU	0.09 Rf	44.1 AU	13.73%	0.11 Rf	6.4 AU	648.6 AU	9.14%
3	0.39 Rf	1.7 AU	0.45 Rf	28.1 AU	8.77%	0.50 Rf	3.9 AU	1061.2 AU	14.95%
4	0.64 Rf	4.5 AU	0.67 Rf	19.5 AU	6.07%	0.70 Rf	7.2 AU	477.1 AU	6.72%
5	0.73 Rf	6.1 AU	0.76 Rf	25.0 AU	7.77%	0.80 Rf	2.6 AU	678.5 AU	9.56%
6	0.82 Rf	6.6 AU	0.86 Rf	33.6 AU	10.45%	0.90 Rf	18.7 AU	1254.8 AU	17.68%

Table 6: HPTLC peak parameters -Rf values, height and area- of root methanolic extracts from vegetative stage in *W. sominifera*.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	%Area
1	0.00 Rf	5.8 AU	0.02 Rf	135.1 AU	23.26%	0.07 Rf	23.2 AU	3358.0 AU	18.99%
2	0.07 Rf	23.6 AU	0.08 Rf	41.5 AU	7.15%	0.12 Rf	2.0 AU	596.7 AU	3.37%
3	0.32 Rf	0.0 AU	0.39 Rf	16.6 AU	2.85%	0.40 Rf	5.6 AU	490.3 AU	2.77%
4	0.44 Rf	6.4 AU	0.48 Rf	25.0 AU	4.30%	0.50 Rf	14.4 AU	671.9 AU	3.80%
5	0.54 Rf	25.7 AU	0.58 Rf	35.1 AU	6.04%	0.60 Rf	21.4 AU	1133.3 AU	6.41%
6	0.61 Rf	23.3 AU	0.67 Rf	78.6 AU	13.54%	0.71 Rf	59.2 AU	3558.1 AU	20.12%
7	0.71 Rf	59.4 AU	0.76 Rf	128.4 AU	22.12%	0.80 Rf	10.4 AU	4869.5 AU	27.53%
8	0.81 Rf	9.9 AU	0.84 Rf	29.8 AU	5.14%	0.86 Rf	20.4 AU	730.1 AU	4.13%
9	0.89 Rf	18.0 AU	0.95 Rf	46.5 AU	8.00%	0.96 Rf	42.7 AU	1555.1 AU	8.79%
10	0.96 Rf	43.2 AU	0.96 Rf	44.0 AU	7.59%	1.00 Rf	1.1 AU	723.6 AU	4.09%

Table 7: HPTLC peak parameters (Rf values, height and area) of methanolic extracts from roots of reproductive stage in *W. sominifera*.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	%Area
1	0.01 Rf	55.3 AU	0.01 Rf	55.3 AU	18.47%	0.05 Rf	0.3 AU	728.6 AU	8.70%
2	0.13 Rf	6.0 AU	0.17 Rf	32.9 AU	10.99%	0.21 Rf	11.4 AU	978.5 AU	11.69%
3	0.33 Rf	2.6 AU	0.39 Rf	19.2 AU	6.43%	0.40 Rf	15.7 AU	467.9 AU	5.59%
4	0.40 Rf	15.8 AU	0.41 Rf	18.3 AU	6.11%	0.44 Rf	4.1 AU	394.9 AU	4.72%
5	0.64 Rf	11.3 AU	0.70 Rf	59.6 AU	19.93%	0.74 Rf	39.1 AU	2786.5 AU	33.29%
6	0.76 Rf	41.7 AU	0.78 Rf	71.4 AU	23.86%	0.82 Rf	0.2 AU	1785.0 AU	21.32%
7	0.91 Rf	6.7 AU	0.94 Rf	42.5 AU	14.22%	0.99 Rf	0.1 AU	1229.8 AU	14.69%

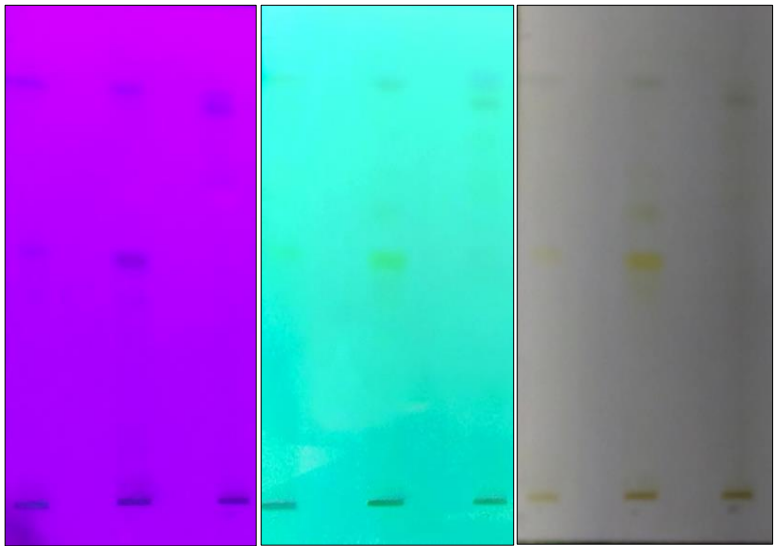

Fig 1: HPTLC chromatograms of leaf methanolic extracts observed under UV 366 nm, UV 254 nm, and normal daylight. Methanolic extracts of 10 µl leaf samples were applied in the lanes (i) seedling (ii) vegetative and (iii) reproductive stages respectively.

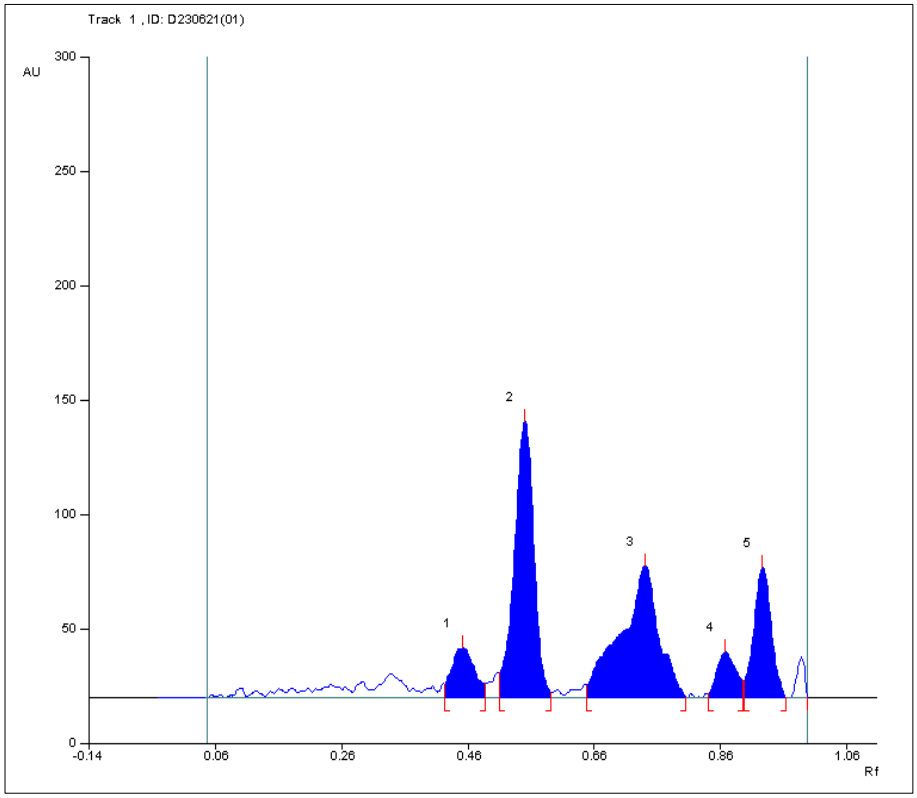

Fig 2: HPTLC densitometric chromatogram at 450 nm of leaf methanolic extracts from seedling stage in *W. sominifera.*

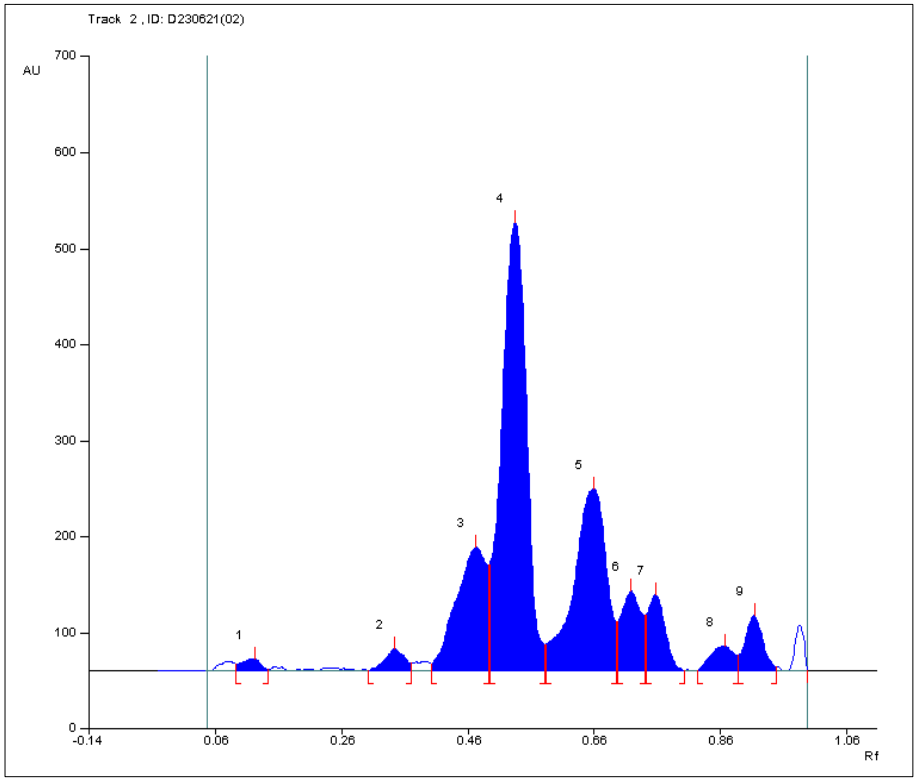

Fig 3: HPTLC densitometric chromatogram at 450 nm of leaf methanolic extracts from vegetative stage in *W. sominifera*.

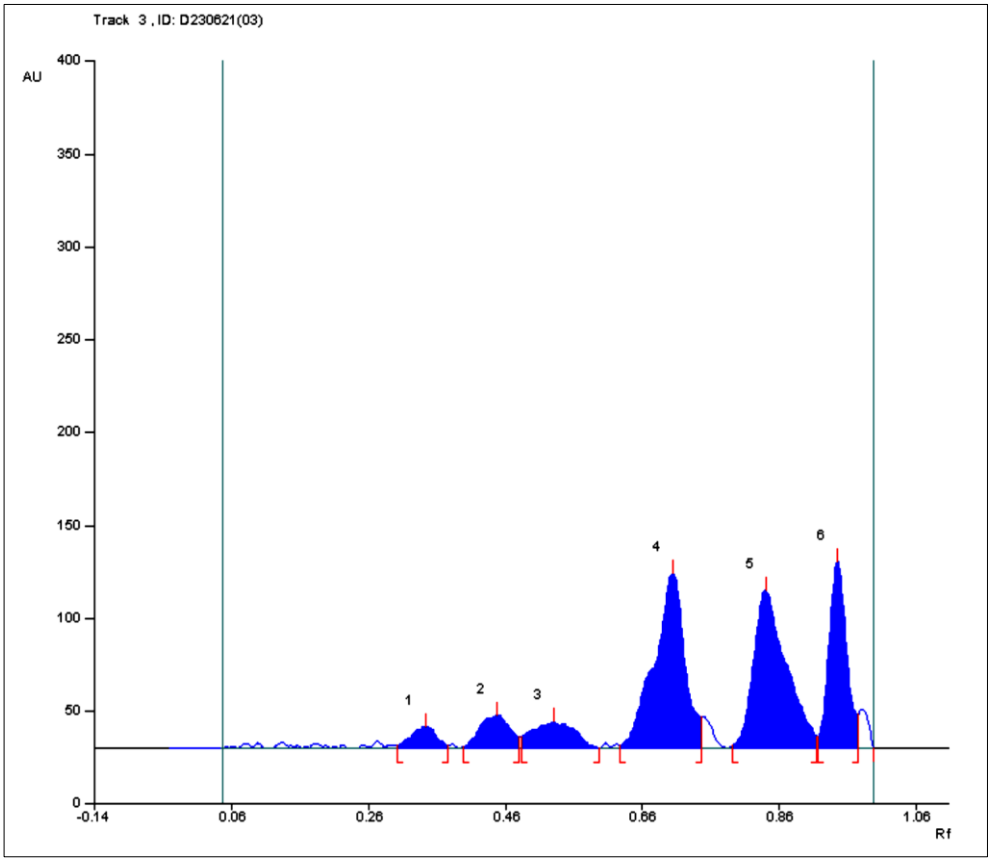

Fig 4: HPTLC densitometric chromatogram at 450 nm of methanolic extracts from leaf of reproductive stage in *W. sominifera*.

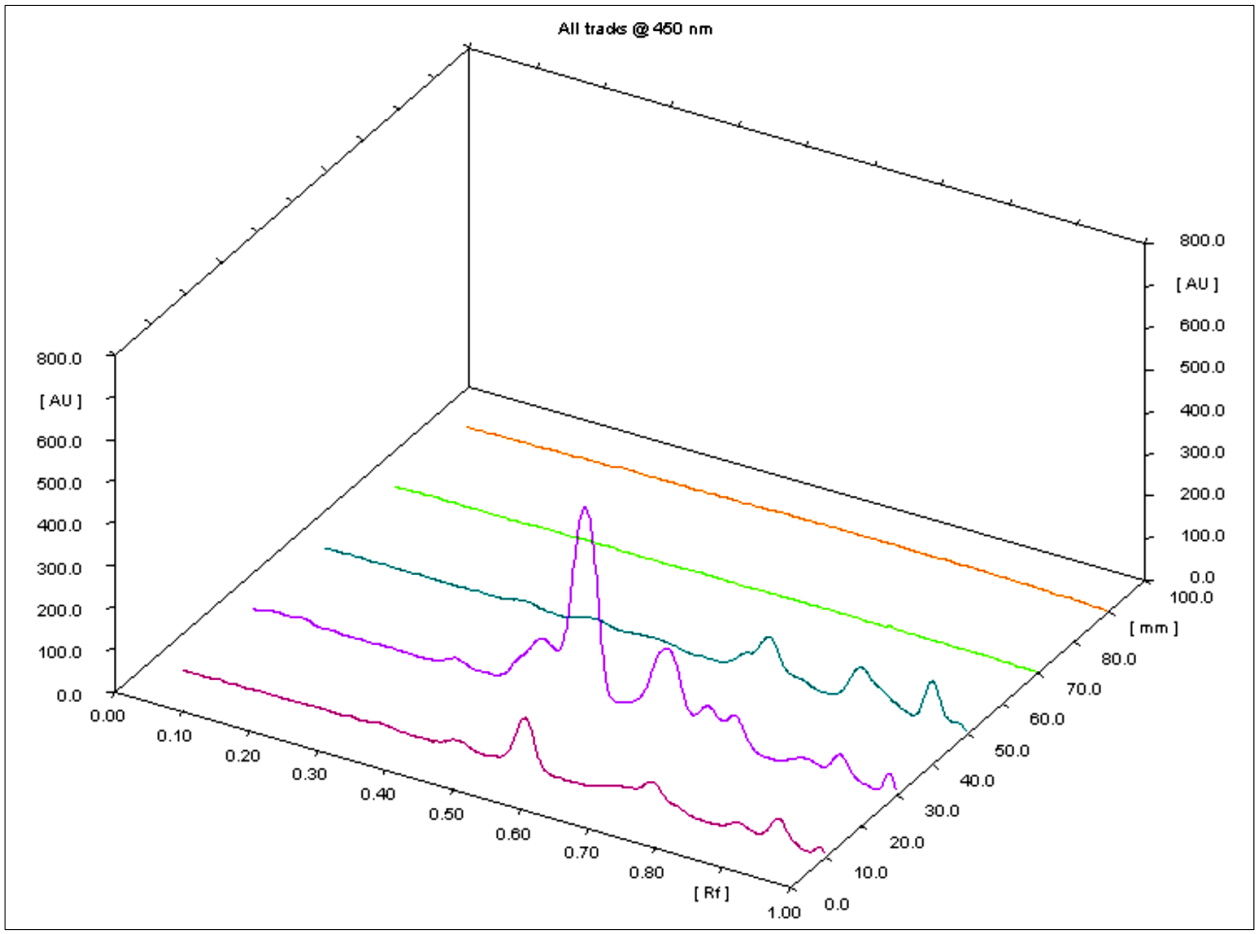

Fig 5: 3D densitogram at 450 nm of methanolic extracts from leaf of seedling, vegetative and reproductive stages in *W. sominifera*.

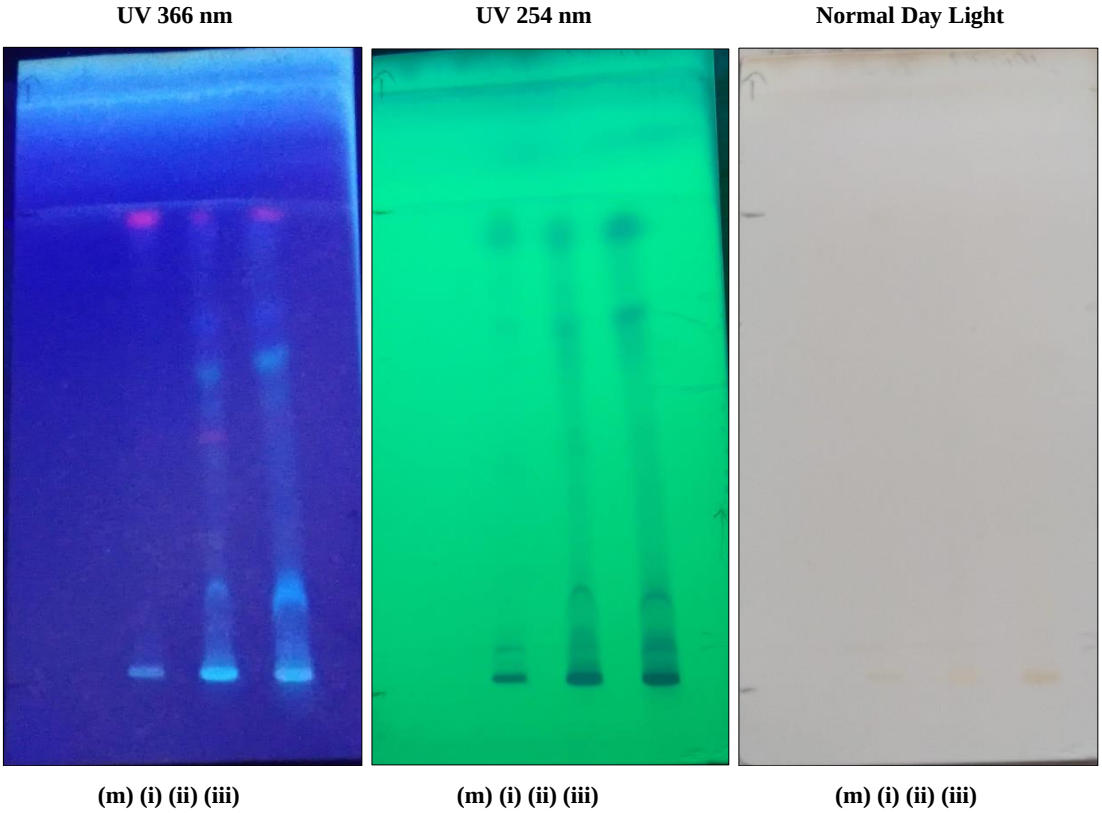

Fig 6: HPTLC chromatograms of methanolic extracts of roots observed under UV 366 nm, UV 254 nm, and normal daylight. Lanes representing (m) methanol blank (i) seedling (ii) vegetative and (iii) reproductive stages with 10 µl equal volume of applied root methanolic extracted samples.

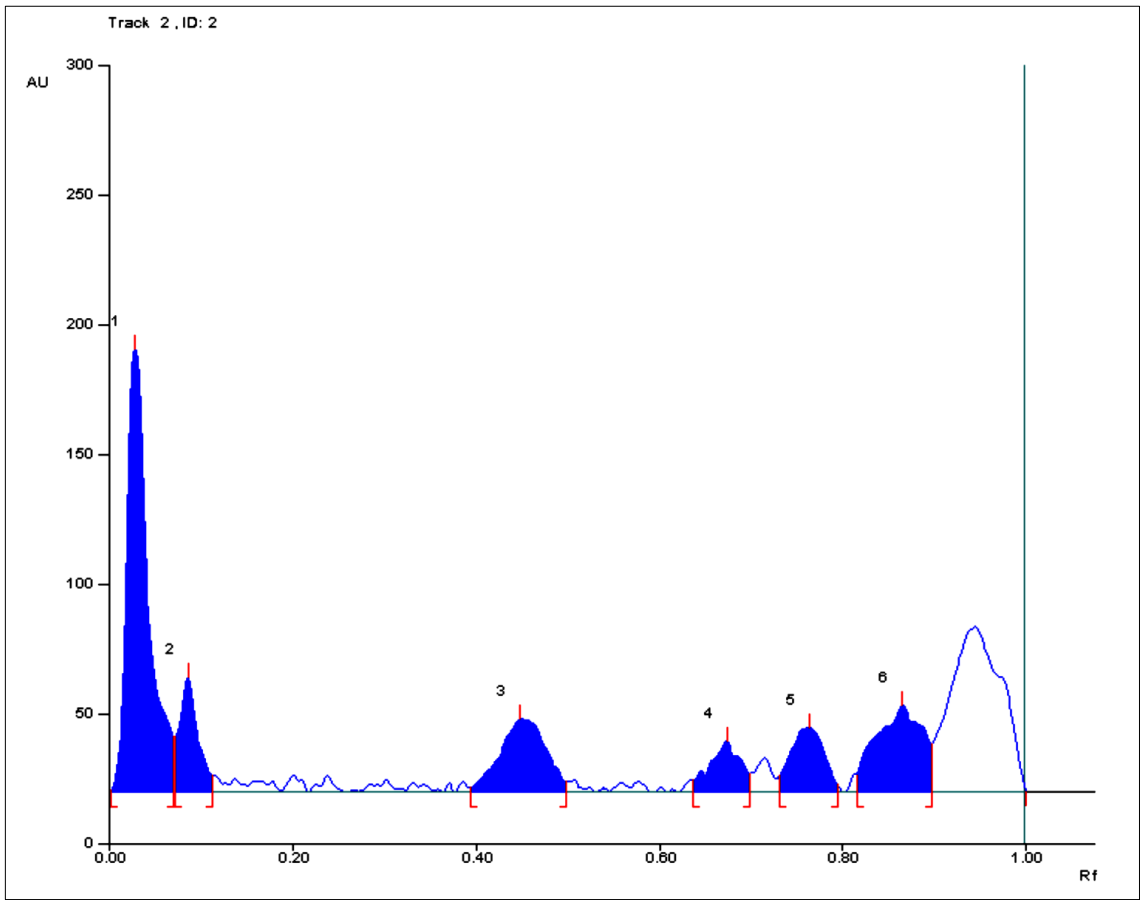

Fig 7: HPTLC densitometric chromatogram at 450 nm of methanolic extracts of root from seedling stage in *W. sominifera.*

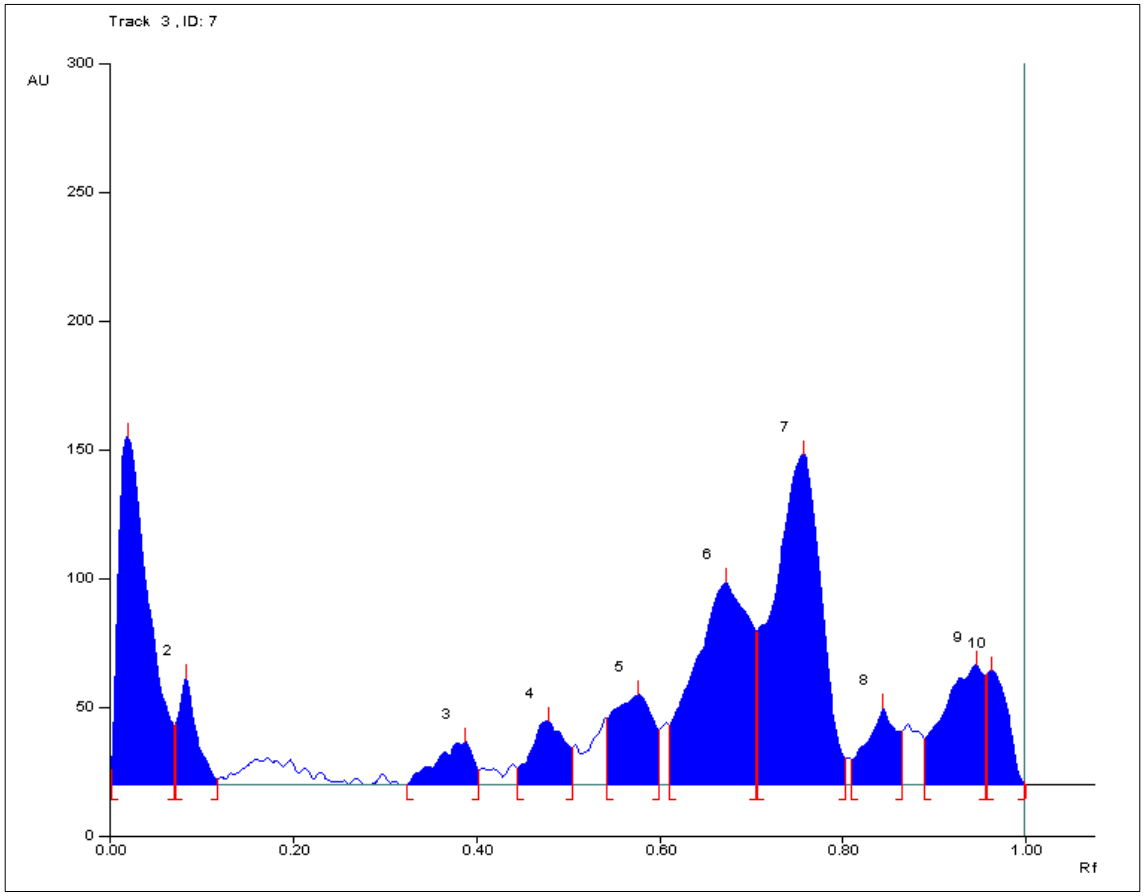

Fig 8: HPTLC densitometric chromatogram at 450 nm of root methanolic extracts from vegetative stage in *W. sominifera*.

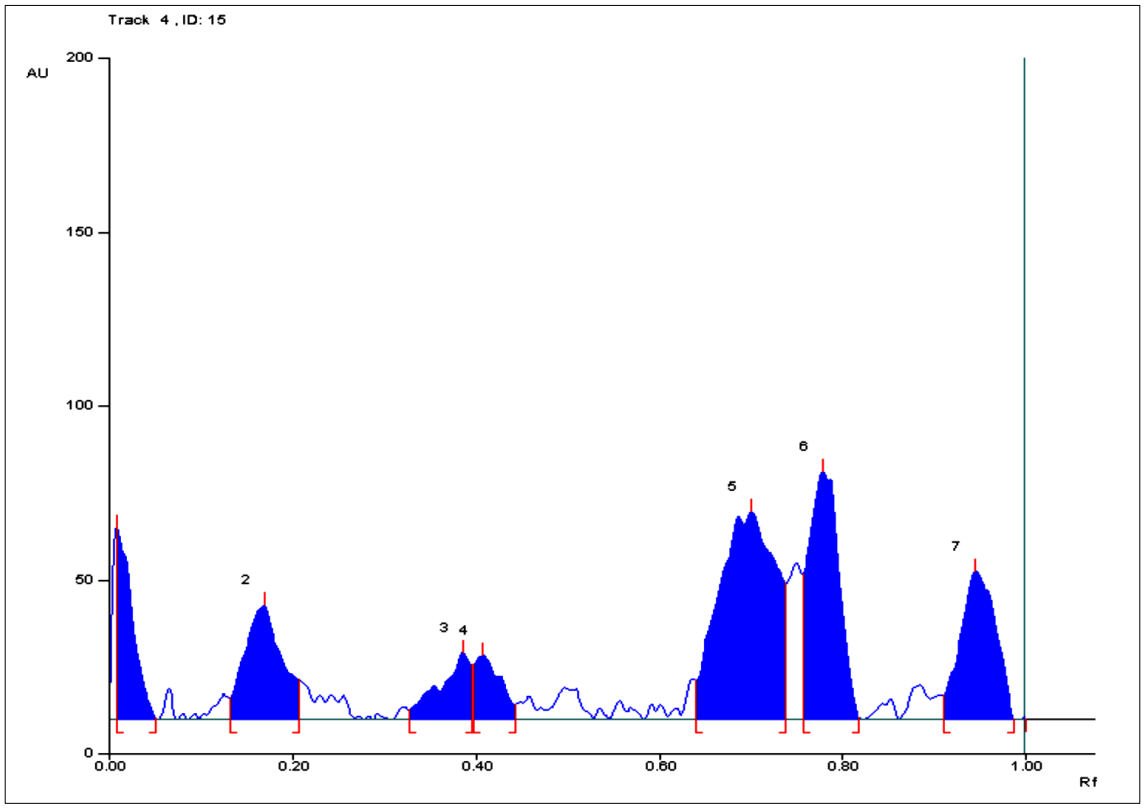

Fig 9: HPTLC densitometric chromatogram at 450 nm of root methanolic extracts from reproductive stage in *W. sominifera*.

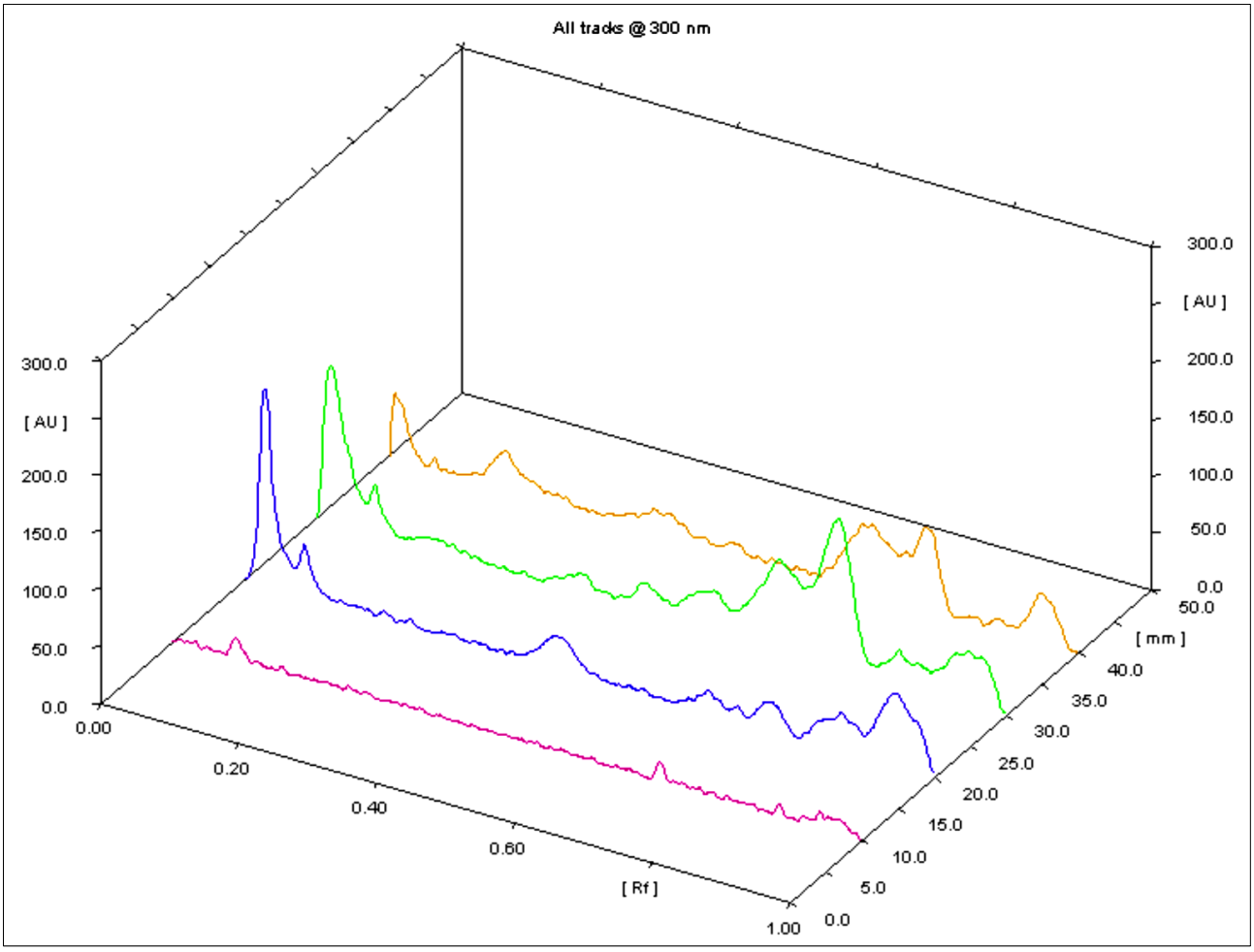

Fig 10: 3D densitogram at 450 nm of root methanolic extracts from seedling, vegetative and reproductive stages in *W. sominifera*.

5. Conclusion

W. somnifera, a well-known herbal medicinal plant used for various ailments, was explored for the presence of variation in the phytochemical constituents in leaf and roots of seedling, vegetative and reproductive stages. In seedling stage, the number of bands in chromatograms were less than vegetative and reproductive stages. In vegetative stage, the number of bands are comparatively more than reproductive stage. The comparative HPTLC fingerprinting of leaves and roots showed the highest number of peaks at vegetative stage, whereas the least number of peaks were shown at seedling stage. Both leaf and root showed higher peak areas in reproductive stage and least concentrations in vegetative stage. Further fractionation and evaluation of phytochemical constituents can reveal medicinal properties attributed with respect to the developmental stages in ashwagandha.

6. Acknowledgements

LL acknowledges National Fellowship for Higher Education of ST Students (NFST), Ministry of Tribal Affairs - Gover nment of India for fellowship. AS acknowledges Rashtriya Uchchatar Shiksha Abhiyan (RUSA) 2.0 Program, under Ministry of Human Resources Development, Gover nment of India for funding the research. AS also acknowledges funding by CAS, DST-PURSE-II, UPE-FAR and DST-FIST. Authors also thankfully acknowledge NISHKA Research Pvt, Ltd., Hyderabad for providing necessary facilities to carry out the present work.

7. Conflicts of Interest

The authors declare no conflicts of interest.

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